



Expression, production and purification of proteinases from microbes

J. Rajamurugan^{1*}, B. Annadurai²

*Corresponding author:

J. Rajamurugan

¹Department of plant science,
Bharathidasan Government
College for Women,
Puducherry – 605 003, India.
Corresponding author: Mobile:
91+9443948791, E-mail:

rajamurugan.bgcw@gmail.com

²Department of Plant Biology
and Biotechnology, Abdul
Hakeem College,
Melvisharam, Vellore,
India

Abstract

Screening and expression of protease producing 66 strains of different microbes were obtained from the various places in Chennai, Vellore, Tamilnadu, India. The isolates were positive on tyrosin caesin nitrate agar (1%) and thus are selected as protease producing strain. 11 of them belong to miscellaneous microbes. The microbial growth is revealed by the mycelial dry weight determination. Maximum growth is observed in the case of *Bacillus* (0.78 mg). Equally, the maximum growth is observed in *Aspergillus* sp. (0.064 mg), *Fusarium* sp. (0.62 mg) and *Penicillium* sp. (0.62 mg). Finally the enzyme protease was purified by column chromatography. The protein was characterized using SDS-PAGE. Maximum protein content is observed in the case of *Alternaria* sp. (0.902mg) and *Penicillium* sp. (0.624 mg). Maximum proteinase content is observed in *Aspergillus* sp. (0.866mg). This results showed that microbes under study is a good producer of extra cellular protease, which can be beneficial for industries.

Introduction

Enzymes are delicate protein molecules necessary for life. Proteinases are one of the most important industrial enzymes found in wide variety of microorganisms. They are molecules of relatively small size, spherical structures that catalyze the peptide bond cleavage in proteins [1]. These enzymes are important in a number of biological processes viz regulation of metabolism [2]. Gene expression, pathogenicity and biological application. It also find application in leather industry, food industry, pharmaceutical and bioremediation process [3,4]. The largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing [5], waste processing industries [6]. Panicker et al, [7] studied the purification and characterization of serine proteases from mature coconut endosperm, while characterization of asparaginyl proteinase was investigated by Oliver et al [8]. The present attempt was aimed to

compare the changes in the production and activity of these useful enzymes proteinases from a number of different microorganisms

Materials and Methods

Collection and isolation of sample

About 66 strains of bacteria, fungi, etc, were obtained from the culture collection centre of Botany Department of CAH college of Melvisharam, CAS Botany, University of Madras, Dept of Biotechnology CLRI and PG. Extension Centre, Vellore and other Microbiology Laboratories nearby. A few strains were isolated from the air microflora of Kancheepuram and cultured in Tyrosin caesein nitrate agar [9].

Mycelial dry weight determination

Mycelial mats formed from microbial cultures were filtered with coarse grade glass filters with water and transferred to a previously weighed

filter paper and dried at 70° C overnight and cooled at room temperature in a desicator and weighed to a constant weight correct to the mg values given in the tables are mean of triplicate values [10-12].

Preparation of Crude extract

The microorganisms along with the culture medium were triturated in a mortar with acid-washed sand. This was centrifuged and the supernatant was collected. The residue was triturated again with distilled water, centrifuged and the supernatant was mixed with the previous extract. The total liquid collected was made up to a known volume and analyzed for proteolytic activity.

Estimation of Protein

Total protein was determined by the method of Lowry et al., (1951) using BSA as the standard. Sample containing 50-100µg protein were mixed with 5ml of alkaline copper solution (freshly prepared with 50 ml of 1% Na₂CO₃ in 0.1 N NaOH and 1 ml of 0.5% CuSO₄ · 5H₂O in 1% oitassuyn tartrate) and kept for 10 mins at room temperature. It was then mixed with 1 ml of Folin-Ciocalteau's phenol reagent and allowed to stand for 30 mins and the blue color developed was read at 640 nm.

Determination of Proteinase enzyme

Proteinase activity was estimated according to the method of Kurnitz [13] using case in as the substrate. In this method, a protein substrate is subjected to enzymatic hydrolysis. The tyrosine liberated by the hydrolysis is quantitatively estimated by measuring the absorbance at 275 nm. The amount of tyrosine liberated is directly proportional to the enzyme activity.

1ml of casein solution (1% w/v in 0.1 m Tris-HCl buffer pH 9.0) was mixed with 0.5 ml of suitably diluted enzyme solution and incubated at 45 C for 10 min. 50% TCA was then added to arrest the enzyme action. The mixture was warmed and filtered through whatman no.1 filterpaper. The filtrate was read at 275 nm. A control was run in

an identical manner except that the enzyme was added after the addition of TCA

Enzyme Purification

Ammonium Sulphate Precipitation

The organism was grown for 48 hours as described previously. The cells were separated by centrifugation (10 000 rpm, 15 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1M Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer. Further purification of dialyzed protein was done by column of Sephadex G-200, SDS-PAGE

The elution profile of protease by Sephadex G-200 column Chromotography showed the dialyzed ammonium sulphate precipitates loaded on to a DEAE Sephadex G 200 column. Proteins eluted are collected and tested for specific protease activity. The absorption peaks at 280 nm were observed. The column purification shows greatest activity of enzyme. Eluted samples from Sephadex column where run on a 12% SDS page stained with silver. The band obtained with the molecular weight of 60,000 Da as protease activity band.

Results

Tab.1 shows the mycelial dryweight, protein content and proteinase activity of different microorganisms in culture filtrate. The growth is revealed by the mycelial dryweight determination (Fig.1). Among 66 microfloras, Alternaria, Aspergillus, Bacillus, Fusarium, Mucor and Penicillium are growing very well in this media. Maximum growth is observed in the case of Saccharomyces sp.(3.76 mg), Tritirachium sp. (3.17 mg), Bacillus sp. (0.78 mg, p<0.001) equally, the maximum growth is observed in Aspergillus sp. (0.064 mg), Fusarium sp. (0.62 mg) and Penicillium sp. (0.62 mg). The growth is also significant in the case of Alternaria, Mucor and Rhizopus sp. Other microfloras show in significant growth. The protein content in

different culture filtrates of various microorganisms. The protein content, as maximum in the case of *Alternaria* sp. (0.902, $p < 0.001$). Equally maximum protein content is observed in the case of *Mucor* sp. *Rhizopus* sp. (0.867mg, $p < 0.001$) and *Penicillium* sp. (0.624 mg). Equally, maximum amount of protein is observed in the culture filtrate of *Aspergillus* sp. (0.514), *Bacillus* sp. (0.162) and *Fusarium* sp. (0.399) and 0.548) other sp. Shows less amount of protein in the culture filtrates. Amongst various microorganisms, *Aspergillus* sp. Secrete maximum extracellular proteinase content in the culture filtrate (0.866 mg). Equally, good amount of proteinase is secreted extracellularly by *Rhizopus* sp. (0.718 mg) and *Bacillus* sp. (0.692 mg). The maximum secretion of proteinase is also observed in *Alternaria* sp. (0.582 mg), *Mucor* sp. (0.589 mg), and *Penicillium* sp. (0.589 mg). *Fusarium* sp. (0.489 mg), other microorganisms also show the extracellular proteinases, but they are not significant.

Discussion

In recent times, microbial proteinases are receiving much attention, because of their increasing applications in many industries like food, chemical, leather and pharmaceutical industries. The new field of enzyme engineering has in a short period of time made striking contribution to industry, medicine, agriculture and in pollution control. Microbial proteinases are obtained from selected strains of yeasts molds and bacteria among the strains, *Alternaria*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor* are producing proteinases. Among the bacterial strains, *Bacillus* is the most useful source for the production of proteinases. Proteinase is particularly suitable for industrial use, as it can be produced economically on a large scale, by controlled fermentation. While concentrating the large scale of production of proteinase enzyme only 66 microfloras have taken for this investigation. Out of which only 5 to 6 microorganisms namely, *Alternaria*, *Aspergillus*, *Bacillus*, *Fusarium*, *Mucor* and *Penicillium* are

turned to be very good sources for the proteinase enzyme production. These microorganisms through cultural methods have suggested for the large-scale production of proteinase enzyme in future.

Acknowledgement

The authors are thankful to Dr. R. Puvanakrishnan, Deputy Director and senior scientist in the Department of Biotechnology Central Leather Research Institute, Adyar, Chennai for the timely help of the purification of proteases in his laboratory.

Reference

1. Polgar, Laszlo. Mechanisms of protease action. CRC Press, Inc., Boca Raton, Florida. 1989;Pgs.43-76
2. Rao MB, Tanksale AM, Ghatge MS and Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 1998;62:597.
3. Anwar A, Saleemuddin M. Alkaline proteases. A Review. Bioresource Technology, 1998; 6:175-183.
4. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbiology and Biotechnology, 2002;59:15-32.
5. Banerjee UC, Sani RK, Azmi W, Soni R. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochemistry, 1999;35:213-219
6. Pastor MD, Lorda GS, Balatti A. Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration rates. Braz. J. Microbiol. 2001;32:1-8.
7. Panicker LM, Usha R, Roy S, Mandal C. Purification and characterization of a serine Protease (CESP) from mature coconut endosperm. BMC Res Notes, 2009;9:2-81.

8. Oliver EM, Skuce PJ, McNair CM, Knox DP. Identification and characterization of an asparaginyl proteinase (legumain) from the parasitic nematode, *Haemonchus contortus*. *Parasitology*. 2006;2:1-8.
9. Annadurai B. Studies on endopolygalacturonase in leafblight disease of onion (*Allium cepa* Linn) caused by *Alternaria cepulae* (Ponnappa) and its interaction with phytohormones. Ph.D. 1989. thesis, University of Madras.
10. Annadurai B, Gopinath D, Palani R. Studies on the role of the cell wall degrading enzymes in leafblight disease of onion (*Allium cepa* Linn.) caused by *Alternaria cepulae*. *Biojournal*, 1998;10:173-178.
11. Annadurai B and Motlag DB. Effect of various carbon sources on production of endopolygalacturonase of *Alternaria cepulae*. *Journal of Ecotoxicology & environmental monitoring*. 2000;10:37-41.
12. Annadurai B, Karunanidhi P, Mahalingam S. Pectic enzymes of *Alternaria cepulae* in leafblight disease of onion J. *Ecobiol*, 1999;11:299-305.
13. Kurnitz M. Crystalline soybean Trypsin Inhibitor, II. General properties. *Journal of General physiology* 1947;30:291-310
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951;193:265-275.